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13. ABSTRACT *(Maximum 200 Words)*

In the third year of this CDA Award we have continued to make progress towards all three Technical Objectives. The most challenging problem is the isolation of novel cDNAs encoding human homologs of yeast DNA damage response genes. Major efforts to isolate cDNAs for *RAD9* and *DUN1* during the previous year have not been successful. In contrast, two hybrid screens have resulted in the isolation of human homologs of *RAD18* and *RAD21*. Thus, the focus over year 3 has been the characterization of the human Rad21 protein in mammalian cells.

We found alterations in expression of human Rad21 mRNA and protein in human breast cancer cell lines. This has lead to development of immunohistochemistry techniques to now expand this research to human breast cancer samples. In Technical Objective 3 we did not see alteration in *RAD21* mRNA or Rad21 protein phosphorylation in human cells exposed to DNA damage. However, we found that induction of the apoptotic pathway (as opposed to DNA damage itself) induces specific cleavage of the human Rad21 cohesin protein. This cleavage product may play a role in signalling subsequent events in apoptosis or result in aneuploidy in cells that survive the apoptotic response.

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PI - Signature 7/6/00 Date

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A. Introduction

The goal of this project is to further define at a molecular level the human gene products required for the normal cell cycle checkpoint response after DNA damage. The checkpoint response is a fundamental mechanism by which cells control their cell division cycle after experiencing DNA damage from radiation. This response results in an arrest in the G1, S and G2 phases of the cycle until damage is repaired. This checkpoint response is conserved among eukaryotes including the budding yeast *Saccharomyces cerevisiae*. Human cells have an additional response which results in apoptosis after DNA damage. In our application, we proposed to exploit the conservation between yeasts and humans to isolate human checkpoint genes by large scale complementation screens and homology searches isolating novel human cDNAs which can complement yeast G2 checkpoint mutant strains. Subsequent Technical Objectives were directed towards understanding the structure and expression of these genes in both normal and malignant mammary cells. The final Technical Objective was to assay changes in expression of these genes after DNA damage. In this report we detail progress in the second year of this award towards all three objectives. This grant is co-funded with a companion IDEA Award for the PI, Dr. Sharon Plon (grant #DAMD17-98-1-8281).

B. Progress toward completing the proposed Technical Objectives.

RESULTS

Technical Objective 1 - Isolation of additional human G2 checkpoint genes.

a. **Complementation Assay:** As previously described, this work comprised the first year of the IDEA award and was not a major focus over the last year of the grant. The characterization of the human *RAD21* cDNA obtained from a two-hybrid screen in year 1 has been a major focus of the second year of this grant.
b. As described in the year 1 progress report, a second approach to isolating human checkpoint genes utilized homologous regions between evolutionarily distant species (*S. cerevisiae and S. pombe)* to develop degenerate PCR based primers. For example, a fission yeast homolog of *RAD9* named *rhp9* was published. Alignment of those sequences reveals areas of homology that may suggest conserved regions of the protein. One such area is in the carboxy terminus consistent with the known BRCT domain. Although initially we made a major effort to develop a series of degenerate PCR primers to these regions the amplified sequences obtained did not demonstrate additional regions of homology to the *S. cerevisiase RAD9* or *DUN1* genes. We proposed to try direct amplification from human mammary cDNA and mammary carcinoma cDNA in order to prevent any bias against long messages or "unclonable" sequences that might not be represented in a cDNA library.

   During year 3, amplification was performed on multiple human cDNA samples including normal mammary cells, breast carcinoma cell line and ovarian cDNA. PCR products were cloned and sequenced. No sequences obtained provided evidence for additional regions of homology between the isolated sequence and the yeast *RAD9* or *DUN1* genes respectively. Thus, the

use of direct cDNA sources from either benign or malignant mammary cells did not result in isolation of novel human cDNAs for these checkpoint genes. As the human genome project nears completion we continue to search for human homologs of these genes. To date, no cDNAs with significant homology (outside the BRCT domain) have been isolated for *RAD9* and *DUN1*. Thus, these two genes may not have a direct homolog in the human genome, but their functions may be carried out by other types of gene products e.g., p53 in the case of Dun1.

Technical Objective 2A – Checkpoint gene structure and expression in human breast cancer cell lines.

As accomplished during years 1 and 2 of this grant, RNA, DNA and protein were derived from eight human mammary derived cell lines, MCF10A, MCF7, MDA-MB-157, MDA-MD-231, MDA-MB-136, BT-20, HBL100 and SKBR-3 grown in culture under controlled conditions. Analysis of expression of *RAD21* reveals increased expression at the RNA level in breast cancer cell lines, specifically MDA-MB-436 and SK-BR-3 in comparison to the HMEC controls.

We then pursued the development of reagents in order to study the expression of human Rad21 in mammary cell lines and breast cancer samples. A polyclonal antibody was obtained towards the end of year 2. Over year 3, we have obtained a large stock of this anti-sera and obtained affinity purified antibody that can be used in immunohistochemistry and immunoflourescence experiments. We also proposed to create a monoclonal antisera for additional studies (eg, immunoprecipitation with polyclonal antisera followed by Western blotting with monoclonal antibody). Monoclonal antibody which recognizes the human Rad21 protein has been obtained by immunization with a GST-Rad21 fusion protein. The monoclonal is functional in both Western blot and immunoprecipitation assays.

We have performed Western blots analysis of the cell lines listed above with the anti-Rad21 polyclonal antibody (figure 1). Expression of Rad21 is variable in human breast cancer cell lines. In particular, the level of Rad21 protein is elevated in MCF-7 and SK-BR-3 cell lines in comparison to MCF10F cells. In contrast Rad21 protein is absent in the BT-20 cell line suggesting a possible mutational mechanism. These results provide a rationale for further analysis of Rad21 protein expression in human breast cancers with known levels of aneuploidy.

In order to accomplish this last goal, we are collaborating with the newly created Baylor Breast Cancer Center (Kent Osborne, Director). They have now tested the anti-Rad21 antibody on a pilot sample of breast cancer samples and optimized the protocol to provide excellent detection by immunohistochemistry after antigen retrieval. This protocol will now allow us to systematically determine the expression of Rad21 in breast cancer samples as a function of chromosome number or aneuploidy.

Technical Objective 3 - Determination of Changes in Response to Radiation of a Human Breast Cancer Cell Line upon Expression of Human Checkpoint Genes.

We used cDNA probes and antisera to study Rad21 expression in normal and checkpoint deficient cell lines after DNA damage. *RAD21* mRNA is not up or down

regulated in response to ionizing radiation. Western analysis revealed that after ionizing radiation there was no change in the major bands representing Rad21 in several cell types including the totally checkpoint deficient A-T cells. Thus, we did not see evidence for a change in phosphorylation or expression after ionizing radiation. However, some cell types demonstrated the production of a lower molecular weight band consistent with cleavage.

Further analysis revealed that cells in the early stages of apoptosis demonstrated cleavage of the endogenous Rad21 protein. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of a 64 kD cleavage Rad21 product (figure 2). Identity of this product is confirmed by recognition by affinity purified polyclonal and monoclonal antibody to human Rad21. This product is detected after induction of apoptosis by both DNA damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide treatment and cytokine withdrawal). Induction of apoptosis is assayed by cleavage of the endogenous PARP protein and morphological changes consistent with apoptosis. In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of DNA damage. Addition of caspase inhibitors to the cells blocks the cleavage of Rad21 after an apoptotic signal (figure 3). Given the role of rad21 in chromosome cohesion, the cleavage product may signal subsequent events of apoptosis including DNA degradation. Further evidence for signalling is provided by the finding that the Rad21 cleavage product is translocated to the cytoplasm after cleavage (figure 4).

The finding that Rad21 is regulated during apoptosis (as opposed to DNA damage itself) is a novel finding. This has lead to a new hypothesis that there may be a direct link between the development of aneuploidy (given Rad21's role in chromosome cohesion) and apoptosis.

C. Key Research Accomplishments – Year 3

- Extensive degenerate PCR cloning to obtain human homologs of *RAD9* and *DUN1* completed utilizing cDNA sources from both normal mammary cells and breast carcinoma cells.
- Monoclonal antibody to human Rad21 protein produced.
- Expression of Rad21 protein assayed in human mammary and breast cancer cell lines.
- Development and optimization of technique to assay Rad21 expression in human breast cancers accomplished.
- Identification of Rad21 cleavage as an early step in apoptosis.

D. Reportable Outcomes:

- Work on human Rad21 was presented at:
  - Cold Spring Harbor Cell Cycle Meeting, May, 2000, Cold Spring Harbor New York (abstract attached).
  - DOD Era of Hope Meeting, June 2000, Atlanta GA.
- Monoclonal antibody to human Rad21 protein produced.

- Manuscript describing regulation of Rad21 during apoptosis is in preparation.
- DOD Concept Grant was selected for award to co-Investigator, Debananda Pati, to explore the newly identified link between apoptosis and chromosome cohesion.

E. Conclusions

In the third year of this CDA Award we have continued to make progress towards all three Technical Objectives. The most challenging aspect of this project is the isolation of novel cDNAs encoding human homologs of yeast DNA damage response genes. As reported previously, complementation of the yeast mutant *rad9* have not yielded human cDNAs with significant homology. Major efforts to isolate cDNAs by degenerate PCR strategies for *RAD9* and *DUN1* during the initial years of the grant have not been successful. The expected release of the first draft of the Human Genome Project later this summer should shed further light on whether such homologs exist or other non-homologous genes provide these functions. In contrast, two hybrid screens using known human DNA damage response/cell cycle genes has resulted in the isolation of human homologs of *RAD18* and *RAD21*. Thus, the focus over year 3 has been the characterization of the human Rad21 protein in mammalian cells.

The subsequent objectives are focused on determination of whether cDNAs isolated in genetic screens are altered in expression or structure in breast cancers. As described above we do see alterations in expression of human Rad21 mRNA and protein in human breast cancer cell lines. This has lead to development of immunohistochemistry techniques to now expand this research to human breast cancer samples.

The results of Technical Objective 3 have been most surprising to date. We did not see alteration in *RAD21* mRNA or Rad21 phosphorylation in human cells exposed to DNA damage. However, we did detect specific cleavage of the protein. This has lead to determination that induction of the apoptotic pathway (as opposed to DNA damage itself) induces specific cleavage of the human Rad21 cohesin protein. This cleavage product may play a role in signalling subsequent events in apoptosis or result in aneuploidy in cells that survive the apoptotic response.

Appendix 1 – Abstract from Cold Spring Harbor Laboratories – Cell Cycle Meeting

Abstracts of papers presented at the 2000 meeting on

THE CELL CYCLE

May 17–May 21, 2000

Arranged by

Fred Cross, *The Rockefeller University*
Stephen Elledge, *Baylor College of Medicine*
J. Wade Harper, *Baylor College of Medicine*
Jim Roberts, *Fred Hutchinson Cancer Center*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

CLEAVAGE OF HUMAN Rad21 COHESIN PROTEIN: POTENTIAL ROLE IN EARLY APOPTOSIS

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Department of Pediatrics, Baylor College of Medicine, Houston, TX

Sister chromatid cohesion during DNA replication plays a pivotal role for accurate chromosome segregation in eukaryotic cell cycle. Analysis of Rad21 function in fission yeast and *SCC1/MCD1* in budding yeast have demonstrated that it is required for appropriate chromosome segregation during normal mitotic cell cycles and double strand break repair after DNA damage. In budding yeast sister chromatid separation is promoted by the cleavage of the cohesin sub-unit Scc1 and may involve ubiquitin-mediated proteolysis of regulatory molecules. In a two-hybrid screen for potential targets of human Cdc34 (hCdc34) ubiquitin-conjugating enzyme, we have isolated human Rad21 (hRad21) as an hCdc34 interactor. Transfection studies in mammalian cells have indicated physical association of hCdc34 and hRad21 using co-immunoprecipitation experiments. Level of hRad21 was significantly enhanced in the presence of proteasome inhibitors, indicating the involvement of ubiquitin-mediated proteolysis. In a parallel set of studies to analyze the role of Rad21 in mammalian cells after DNA damage, we have identified a novel regulation of hRad21 protein in apoptosis. Induction of apoptosis in multiple human cell lines results in the early (4 hours post insult) generation of a 64 kDa cleavage hRad21 product. Although Rad21 is a nuclear protein the cleaved 64 kDa product is found in both nuclear and cytoplasmic fractions. Identity of this product is confirmed by recognition by affinity purified polyclonal and monoclonal antibody to hRad21. This product is detected after induction of apoptosis by both DNA damaging agents (ionizing radiation and topoisomerase inhibitors) as well as non-DNA damaging agents (cycloheximide treatment and cytokine withdrawal). In addition, equivalent doses of ionizing radiation in cells which are resistant to apoptosis do not generate this band; thus, it is not a simple byproduct of DNA damage. Given the role of Rad21 in chromosome cohesion, this cleavage product may signal subsequent events of apoptosis including DNA degradation. A role for Rad21 in apoptosis has been further strengthened by identification of a number of genes involved in apoptosis as interactors of hRad21 in a two-hybrid assay. In summary, ubiquitin-mediated proteolysis may play a role in the cleavage of hRad21 during metaphase-anaphase transition. In addition to previously described functions of Rad21 in chromosome segregation and DNA repair, cleavage of the protein is an early event in the apoptotic pathway. These results provide the framework to identify the physiologic role of hRad21 function in the apoptotic response of normal and malignant cells.

Appendix 2 – Figures

Figure 1 – Analysis of expression of human *RAD21* mRNA and protein in a series of human mammary and breast cancer cell lines.
All lines were grown in DFCI media under similar conditions except the HMEC line which requires proprietary media.
Upper panel: Total RNA was electropheresed in agarose-formaldehyde gels and transferred to nylon membranes. Hybridization with ^{32}P labelled probes for both *RAD21* and *GADPH* were done sequentially.
Lower panel: Total protein was electropheresed in SDS- polyacrylamide gels and transferred to PDVF membranes. After blocking polyclonal antibody to hRad21 and β-actin were sequentially hybridized. Development was using the ECL (Amersham) detection system.

Expression of human Rad21 mRNA and protein in Breast Cancer Lines

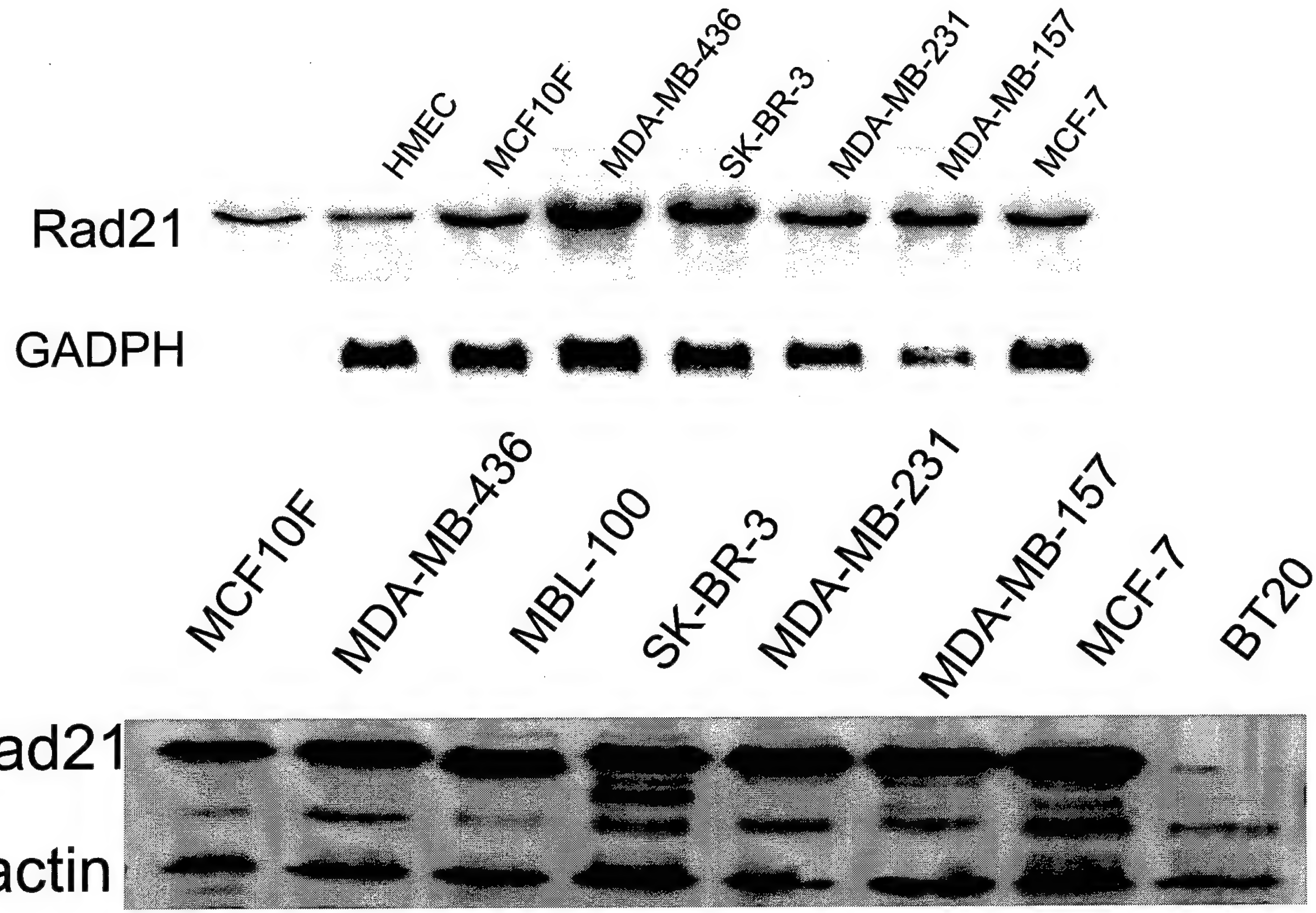

Figure 2 – Detection of a 64kD cleaved product of the human Rad21 protein upon induction of apoptosis for the Jurkat and Molt 4 cell lines.

For each gel either total lysate or immunoprecipitated protein (marked IP) with either a control antibody or anti-Rad21 polyclonal antibody were electropheresed in SDS-polyacrylamide gels. After transfer and blocking the membrane was incubated with monoclonal anti-Rad21 antisera and detected with the ECL system. Apoptosis was initiated by exposure to camptothecin for Jurkat cell line and Etoposide for Molt4 cells. The full length and cleaved forms of Rad21 protein are marked by arrows on the right hand portion of the gel.

Immuno-Precipitation and Western Blot Analysis of Cleaved Rad21 Protein

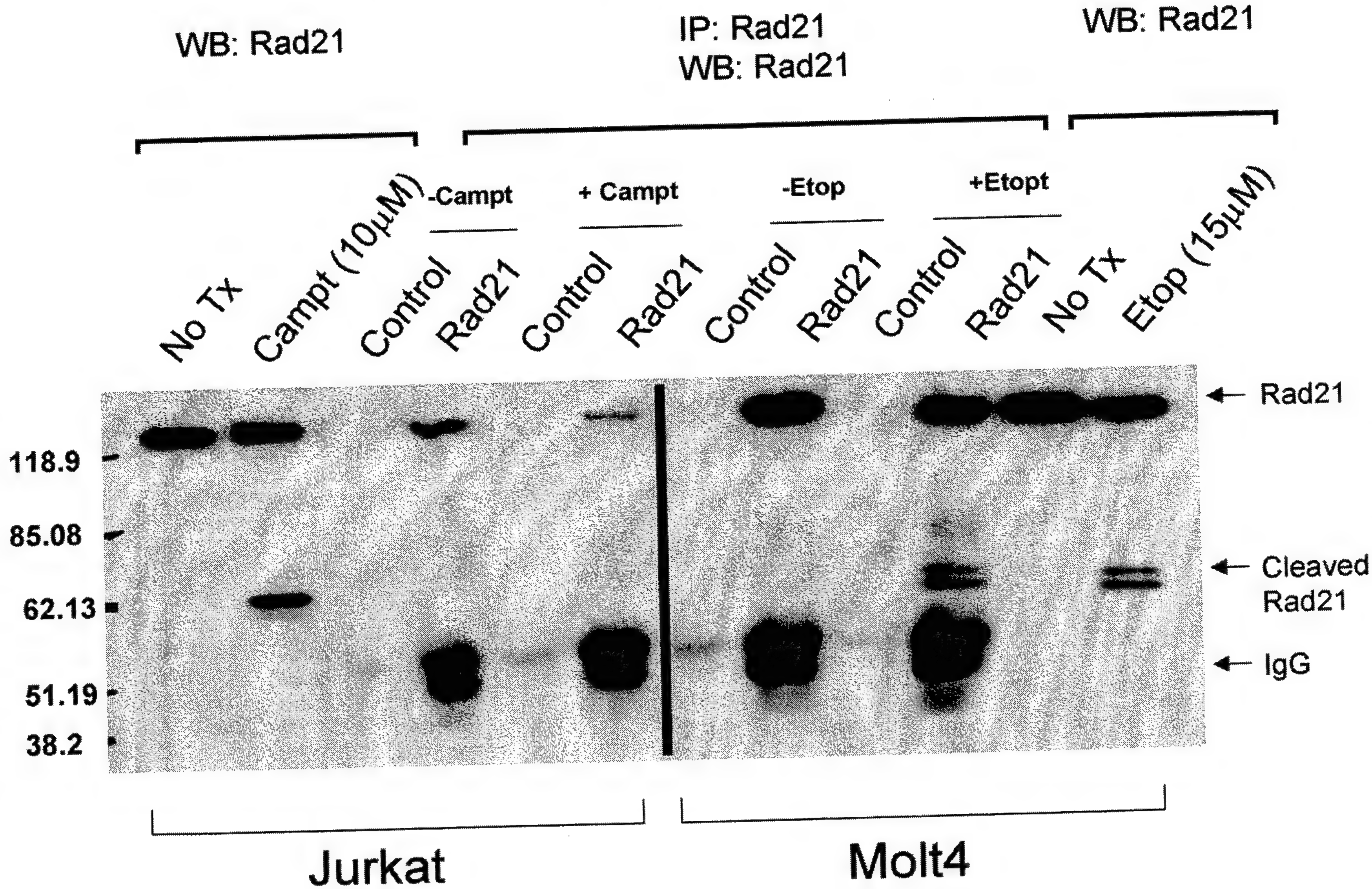

Figure 3 – Inhibition of hRad21 Cleavage by Caspase Inhibitors
Apoptosis was intiated by treatment with etoposide under conditions where Rad21 cleavage occurs as demonstrated in the left two lanes. The third and fourth lanes are duplicate samples where the general caspase inhibitor Z-VAD-FMK were added to the media prior to addition of etoposide. No cleavage of Rad21 is detected under these conditions.

Inhibition of hRad21 Cleavage by Caspase Inhibitor

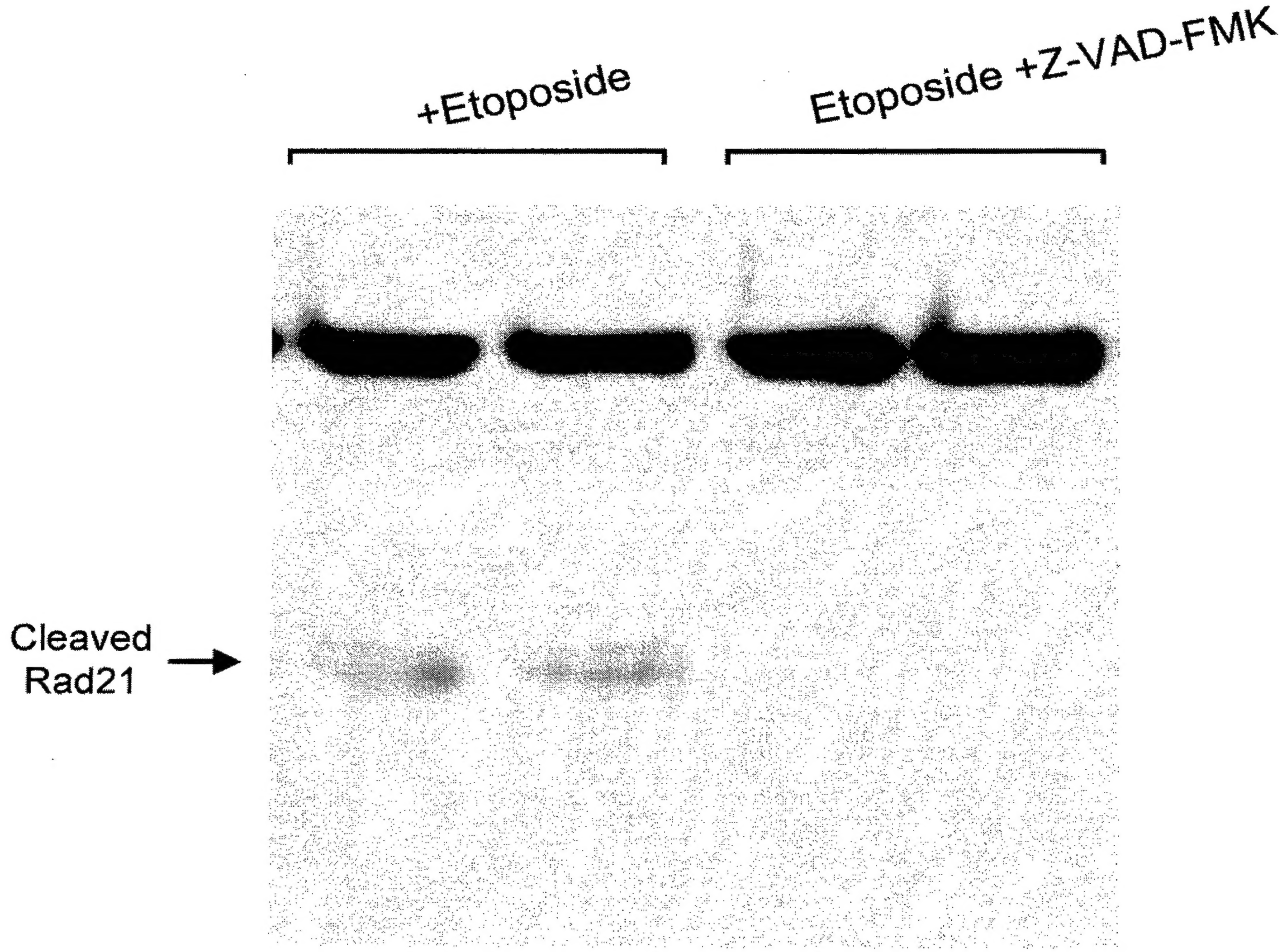

Figure 4 – The Rad21 Cleavage product is Translocated into the Cytoplasm
In order to determine the cellular location of Rad21 a timecourse of Rad21 cleavage after addition of etoposide was performed. At each indicated timepoint the cells were fractionated into a nuclear and cytoplasmic fraction electropheresed in SDS-polyacrylamide gels and transferred for Western blot analysis with anti-Rad21 polyclonal antisera. Although throughout the experiment the full-length Rad21 protein remains nuclear there is clear detection of the 64kD fragment of Rad21 in the cytoplasm by four hours after induction of apoptosis.

Translocation of Rad21 Cleavage Fragment from the Nucleus to the Cytoplasm